UNIVERSITI PUTRA MALAYSIA

DEVELOPMENTAL MORPHOLOGY AND KEY GENES INVOLVED IN EARLY FLOWER DEVELOPMENT IN *ACACIA MANGIUM* WILLD.

KOMALA PONNIAH

FSMB 2003 26

DEVELOPMENTAL MORPHOLOGY AND KEY GENES INVOLVED IN EARLY FLOWER DEVELOPMENT IN *ACACIA MANGIUM* WILLD.

By

KOMALA PONNIAH

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

May 2003

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

DEVELOPMENTAL MORPHOLOGY AND KEY GENES INVOLVED IN EARLY FLOWER DEVELOPMENT IN *ACACIA MANGIUM* WILLD.

By

KOMALA PONNIAH

May 2003

Chairman : Associate Professor Dr. K. Harikrishna

Faculty : Food Science and Biotechnology

Flowering is an important aspect in the life cycle of higher plants, since it signifies the onset of sexual reproduction and the development of seed, grains and fruits. To forest plantations the development of these reproductive structures are a bane since it occurs at the expense of vegetative growth. In Malaysia, *Acacia mangium* which is an important forest plantation species flowers profusely and thus much of its photosynthate is converted to produce reproductive structures. This study was initiated to understand the genetic control of flower development in *A. mangium* with the ultimate aim of controlling this process towards our requirement.

With the help of scanning electron microscopy and light microscopy, early flower development in *A. mangium* was documented. The emerging inflorescence primordium is double-protected by a thick piece of bud scale which in turn lies beneath a stipule-like structure which also protects the phyllode primordium. Flower meristems are formed only after the bracts have been laid down in a spiral pattern along the axis of the inflorescence. The sepals and petals are arranged in alternating pentamerous whorls. Sepals are initiated in a helical fashion in either

direction, with the first sepal varying in position while petals are initiated simultaneously. Development along the axis is slightly staggered with flowers at the base being more advanced than flowers at the tip of the inflorescence, but prior to anthesis all flowers are synchronized developmentally.

AML, the *A. mangium* equivalent of LEAFY (LFY), a floral meristem identity gene, was isolated from young inflorescences. AML is 77% identical to UNIFOLIATA (UNI), the homologue of LFY from pea. Its expression was detected mainly in shoot apical meristems (SAM) from mature flowering trees and young inflorescences. The AML gene is single copy in the *A. mangium* genome and has two introns. AAL, the *A. auriculiformis* equivalent of LFY is 98% identical to AML and 76% identical to UNI. Both AML and AAL formed a clade with UNI to represent the legumes in a phylogenetic tree constructed from various LFY homologues.

Two MADS-box genes have also been cloned from *A. mangium*. *AmMADS1*, the first MADS-box gene reported in a Mimosoideae was isolated from the SAM of mature flowering trees. *AmMADS1* is very similar in sequence to *BpMADS5* from silver birch with an identity of 63% and is expressed in all reproductive tissues including young pods with the highest expression in developing inflorescences. *AmMADS1* is also expressed in roots. Sequence analysis and phylogenetic analysis show that *AmMADS1* belongs to the SQUA subgroup of the MADS-box family of transcription factors.

The second MADS-box gene, *AmMADS2*, also isolated from the SAM of mature flowering trees, is 55% identical at the amino acid level to a MADS-box gene from *Pimpinella brachycarpa* and 50% identical to *AGL20* (*SOC1*) from *Arabidopsis thaliana*. *AmMADS2* is found to be expressed exclusively in SAM, with the vegetative SAM showing a higher expression than SAM from mature flowering trees. Phylogenetic analysis shows that *AmMADS2* is a member of the TM3 subfamily and groups specifically with SOC1 and SaMADSA from *Sinapis alba*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERKEMBANGAN MORFOLOGI DAN PENGLIBATAN GEN UTAMA DALAM PERKEMBANGAN AWAL BUNGA *ACACIA MANGIUM* WILLD.

Oleh

KOMALA PONNIAH

Mei 2003

Pengerusi : Profesor Madya Dr. K. Harikrishna

Fakulti : Sains Makanan dan Bioteknologi

Proses pembungaan merupakan satu aspek penting dalam kitar hidup tumbuhan berbunga kerana ia menandakan permulaan pembiakan seksual. Selain daripada itu proses ini juga menghasilkan biji benih, bijirin dan buah. Namun demikian, ladang-ladang perhutanan tidak melihat hasil pembungaan ini sebagai sesuatu yang menguntungkan. Malahan, hasil pembungaan ini dikatakan membantut perkembangan vegetatif yang membawa akibat negatif kepada hasil perhutanan.

Acacia mangium merupakan spesis ladang perhutanan penting di Malaysia. Spesis ini berbunga dengan lebatnya sepanjang tahun dan sebahagian besar hasil fotosintesis digunakan untuk perkembangan bunga dan buah. Kajian ini dilakukan bagi memahami perkembangan pembungaan *A. mangium* dari segi genetik dan seterusnya menggunakan maklumat yang diperoleh untuk mengawal proses tersebut mengikut kehendak kita.

Perkembangan awal pembungaan *A. mangium* telah didokumentasikan dengan menggunakan mikroskop cahaya dan elektron. Primordia infloresen dilindungi

oleh lapisan tebal "bud scale" yang seterusnya dilindungi pula oleh struktur berbentuk stipul. Kehadiran meristem bunga hanya dilihat selepas pembentukan "bract" di sepanjang paksi infloresen. Lima keping sepal dan lima keping petal diatur secara alternat. Sepal terbentuk dalam urutan heliks, samada arah ikut jam atau lawan jam dan kedudukan sepal pertama biasanya tidak tetap, manakala petal pula dibentuk serentak. Perkembangan bunga di sepanjang paksi infloresen adalah tidak seragam: bunga di bahagian pangkal infloresen biasanya lebih matang daripada bunga di bahagian hujung. Walau bagaimanapun, sebelum antesis, semua bunga biasanya dalam tahap perkembangan yang sama.

Homolog LEAFY (LFY), salah satu gen identiti meristem bunga, dari *A. mangium* telah dipencilkan dari infloresen muda dan diberi nama AML. AML adalah 77% serupa dengan UNIFOLIATA (UNI), homolog LFY dari kacang pea. Ekspresi gen ini hanya ketara dalam meristem pucuk daripada pokok matang yang berbunga dan juga infloresen muda. Gen AML ini merupakan salinan tunggal dalam genom *A. mangium* dan mengandungi dua intron. AAL, homolog LFY dari *A. auriculiformis* pula adalah 98% serupa dengan AML dan 76% serupa dengan UNI. AML dan AAL bersama-sama UNI membentuk satu kumpulan bagi mewakili tumbuhan legum dalam pokok filogeni yang dibentuk dari berbagai homolog LFY.

Dua gen kotak-MADS juga telah diklonkan dari *A. mangium*. AmMADS1, gen kotak-MADS pertama dari Mimosoideae dipencilkan dari meristem pucuk pokok matang yang berbunga. AmMADS1 mempunyai identiti sebanyak 63% dengan BpMADS5 daripada "silver birch". Ekspresi gen ini dilihat dalam semua tisu pembiakan termasuk buah atau pod muda dengan ekspresi paling ketara dalam

infloresen muda. Selain daripada itu, gen ini juga diekspres dalam akar. Analisis urutan asid amino dan filogeni menunjukkan AmMADS1 ahli kumpulan SQUA dalam famili gen kotak-MADS.

Gen kotak-MADS yang kedua, AmMADS2 juga dipencilkan dari meristem pucuk pokok yang berbunga. Gen ini mempunyai identiti sebanyak 55% dengan gen kotak-MADS dari *Pimpinella brachycarpa* dan sebanyak 50% dengan AGL20 (SOC1) dari *Arabidopsis thaliana.* AmMADS2 didapati hanya diekspres dalam meristem pucuk. Ekspresi tinggi diperhatikan dalam meristem pucuk vegetatif dan ekspresi yang rendah dalam meristem pucuk pokok berbunga. Analisa filogeni menunjukkan AmMADS2 ahli kumpulan TM3 dan berkelompok secara khususnya dengan SOC1 dan SaMADSA dari *Sinapis alba.*

ACKNOWLEDGEMENTS

Gratitude is the hardest of emotions to express, but nevertheless, I shall try to convey my heartfelt thanks to these people:

To my supervisor, Associate Professor Dr. K. Harikrishna: for his guidance and encouragement and not forgetting the ideas for carrying out this project. To Dr. Sharifah Shahrul Rabiah Syed Alwee: for her constant motivation and support and especially for her friendship and generosity. To Dr. Tan Siang Hee: for his advice and comments.

To the management and staff of TropBio Research Sdn. Bhd.: especially to the Executive Director, Dato' Dr. Salleh Mohd. Nor for providing the opportunity and support to pursue this project. To Dr. Francis Ng for his generosity and for his help in sample collection.

To the Ministry of Science and Technology: for the Malaysian Teaching Company Scheme (MTCS) grant that funded this project, especially to Encik Sakip. To En. Roslan of Graduate School for his help in purchasing and to Mr. Ong from Dept. of Biotechnology for his help in getting the paperwork sorted out.

To my friends and ex-colleagues: Chiew Foan, Zaiton, Dr. Jenni, Jamilah, Annie and Dr. Fong for helping to establish a conducive environment for molecular biology research and providing a supportive network during the working hours. Siew Eng a wonderful friend who is always there to lend a hand and provide constructive input. Meilina for her friendship and generosity, especially for

providing a 'second home'. Zaidah for her help in *in situ* work, Mas, Azizah, Feshah, Rajini, Roslan and Shamsul for their help. Also to Balan and Pooven for their help in collecting samples.

To Ahmed Eldouma of Forestry Faculty for giving access to *A. mangium* and *A. auriculiformis* samples. To Puan Halimah of Forestry Faculty for assistance in histological work and also to the EM staff at Faculty of Veterinary Science for help in SEM work. To Prof. Paula Jameson of Massey for giving me the opportunity to spend some time in her lab and also for insightful discussions.

To my wonderful family: for their love and encouragement and never ending support and faith in me, especially to my mum for providing an excellent example of perseverance. To my husband Steve: for his love, support and wonderful patience and for always being there.

OM NAMA SHIVAYA - TO GOD BE THE GLORY!

I certify that an Examination Committee met on 26th May 2003 to conduct the final examination of Komala Ponniah on her Doctor of Philosophy thesis entitled "Developmental Morphology and Key Genes Involved in Early Flower Development in *Acacia mangium* Willd." in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Suhaimi Napis, Ph.D.
Associate Professor
Department of Biotechnology
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairman)

Harikrishna a/l Kulaveerasingam, Ph.D.
Associate Professor
Department of Biotechnology
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

Tan Siang Hee, Ph.D.
Department of Biotechnology
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

Sharifah Shahrul Rabiah bt. Syed Alwee, Ph.D.
Advanced Biotechnology and Breeding Centre
Biology Division
Malaysian Palm Oil Board
(Member)

J.S. Heslop-Harrison, Ph.D.
Professor
Department of Biology
University of Leicester
United Kingdom
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 9 JUN 2003

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Harikrishna a/l Kulaveerasingam, Ph.D.
Associate Professor
Department of Biotechnology
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairman)

Tan Siang Hee, Ph.D.
Department of Biotechnology
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

Sharifah Shahrul Rabiah bt. Syed Alwee, Ph.D.
Advanced Biotechnology and Breeding Centre
Biology Division
Malaysian Palm Oil Board
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 11 JUL 2003

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

KOMALA PONNIAH
Date: 9/6/03

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
2-BE	ethyleneglycolmonobutylether
AAL	*Acacia auriculiformis* LEAFY
AML	*Acacia mangium* LEAFY
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cDNA	copy DNA
dATP	2'-deoxy-adenosine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTPs	deoxynucleotides
DTT	dithiotrietol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis- (β-aminoethyle ether)
EtBr	ethidium bromide
g	gram

HCl	hydrochloric acid
hr	hour(s)
k	kilo
kb	kilobase
KCl	potassium chloride
L	liter
LFY	LEAFY
LiCl	lithium chloride
M	molar
MADS	MCM1-AGAMOUS-DEFICIENS-SRF
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MMLV	Maurine Moloney Leukemia Virus
MOPS	3-(N-morpholino) propane-sulphonic acid
mRNA	messenger RNA
MW	molecular weight
N	normal
NaCl	sodium chloride
NaOAc	sodium acetate

ng	nanogram
n-j	neighbour-joining
N-terminal	amino terminal
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Poly A(+) RNA	polyadenylated RNA
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
rRNA	ribosomal RNA
rt	room temperature
RT	reverse transcriptase
SAM	shoot apical meristem
SEM	scanning electron microscope
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate buffer
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	Tris-HCl-EDTA
tRNA	transfer RNA
U	unit

UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galatopyranose

CHAPTER 1

INTRODUCTION

The world's wood supply is now shifting from natural forests stands to intensively managed, fast-growing plantations to accommodate the increasing demand for pulp and paper products by a growing worldwide population. *Acacia mangium*, a fast-growing tropical tree, is a commercially important leguminous tree for the pulp and paper industry and is currently a leading tree for plantations in this region. It is estimated that by the year 2004, the Asia Paper and Pulp group will obtain most of its wood from plantations consisting mainly of *A. mangium* (Bayliss, 1998).

Being a tropical perennial with economic importance, *A. mangium* is a good candidate for genetic improvement. With its fast growth and fairly early flowering characteristics, it also makes a good model for tropical forest trees. Apart from that, *A. mangium* has a relatively small genome size of approximately 748 Mbp per haploid genome (Arumuganathan, K., pers. comm.), which is a prerequisite for a model system. Molecular biology tools have been used successfully to genetically transform tree species such as poplar, which is now an established model system for temperate trees.

Engineering reproductive sterility along with other value-added traits to overcome transgene escape into the wild populations is of utmost importance in the

endeavours of genetic improvement of trees. Moreover, blocking the reproductive pathway, which redirects energy resources to vegetative growth, is an appropriate strategy to increase wood yield. On the other hand, induction of early flowering is beneficial in terms of shortening the breeding cycle, which allows for early characterization of transgene inheritance.

The key step in engineering floral sterility or hastening the flowering process in a plant species is the isolation and characterization of floral genes. Great progress has been made in understanding the mechanisms that control the flowering process in day length dependent plants like *Arabidopsis*. In contrast, very little is known about the molecular mechanisms that underlie this process in tropical, day-neutral, perennial plants. Nevertheless, studies have shown that structures and functions of floral genes between highly divergent species remain unchanged across large phylogenetic distances (Weigel and Nilsson, 1995). The isolation and characterization of *Arabidopsis* genes, which are involved in the flowering initiation process, provides an opportunity for us to study the mechanisms that control flowering process in tree species.

Floral initiation involves an array of genes, which play a role in the maturation or phase change in trees. The *LEAFY* (*LFY*) gene from *Arabidopsis* has been demonstrated to be the earliest acting of the floral meristem identity genes. The overexpression of *LFY* in aspen (poplar hybrid) shortened flowering time by 90% in a species that normally takes many years to reach maturity (Weigel and Nilsson, 1995).